CHAPTER 3

MATERIAL AND METHODS

3.1 Place of research and parties involved

The trees observed in this research are located in different places in Stuttgart, Germany. The Oak trees (*Q. robur*) and the Sycamore trees (*P.* x *hispanica*) are located at "Schwieberdinger Straße" at Stuttgart's north close to the suburb Stammheim.

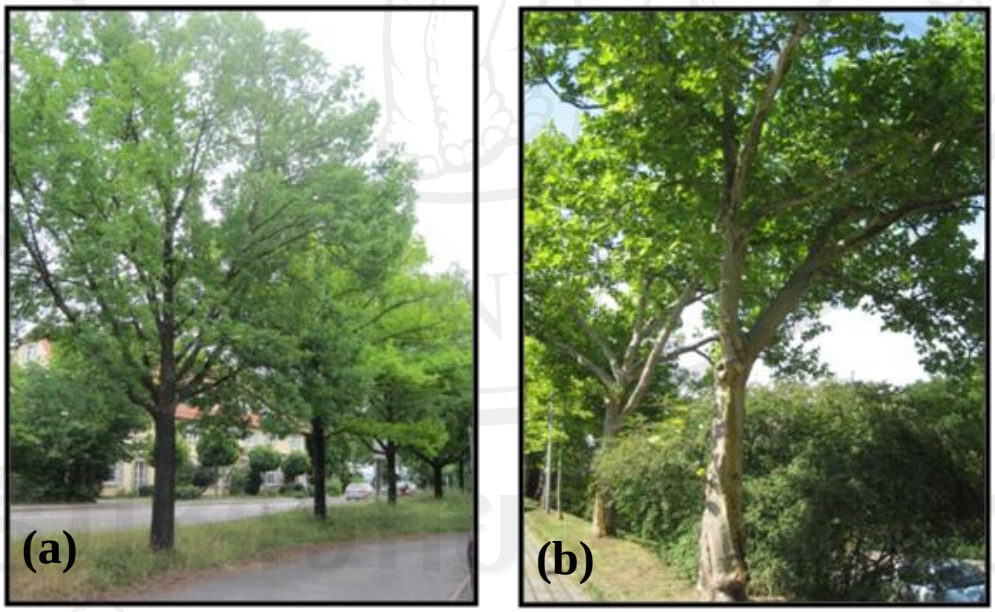

Figure 3.1a, b: Oak trees (a) and Sycamore trees (b) at their location in "Schwieberdinger Strasse"

The Horse chestnut trees (*A. hippocastanum*) are located at “Haußmann Strasse”, east of town center.

Figure 3.1c: Horse chestnut trees at their location in “Haußmann Strasse”

The different research locations were selected in cooperation with the “Gartenbauamt Stuttgart” and the NeemAzal T/S producing company, Trifolio-M GmbH (Wetzlar, Germany).

All data examined during experimental analyses in this research were collected from the above mentioned locations.

3.2 Experimental design and test procedure

On each of the three locations, a representative amount of trees (treated and un-treated) were selected in a random manner. The starting date for the application was determined depending on the target pest phenology and their actual occurrence influenced by weather conditions.

The following table (Tab. 3.1) shows application and leaf collection dates on the research objects.

Table 3.1: Dates for application as well as for leaf collection on tree species

Application of Neem Azal with monitoring of pest and leaf collecting dates:											
	20. April	05. May	18. May	25. May	31. May	15. June	22. June	28. June	12. July	19. July	27. July
Quercus	X	X	X	X	*(removal of nests)*						
Platanus	X	X	X	X	X	X	X	X			
Aesculus			X	X	X	X	X	X	X	X	X

The general idea to apply the Neem compound in a two week interval on tree trunks was given from the NeemAzal-T/S producing company Trifolio-M GmbH. The first day of NeemAzal-T/S application on *Q. robur* and *P. x hispanica* was April 20th. Initial application for the *A. hippocastanum* trees was on May 18th.

Each of the species was supposed to receive six NeemAzal-T/S applications with a delay of two weeks after initial application. This has been realized in the cases of *P. x hispanica* and *A. hippocastanum*, but not for *Q. robur*, since the administration of "Gartenbauamt Stuttgart" decided that the removal of the nests of the Oak processionary moth (OPM) became necessary, due to increased human health risks.

The removal of the OPM nests happened in the first week of June, after three NeemAzal-T/S applications and one leaf collection incident. With the removal of the OPM nests, the experimental analysis on *Q. robur* came to an abrupt end.

For compilation of results on *Q. robur*, only data from the period between first application on April 20th, and nest removal in June, were available. Leaf collection on *Q. robur* happened once on May 25th.

For *P. x hispanica* six applications of NeemAzal-T/S with a time-interval of two weeks could be realized and two leaf collection incidents on May 25th, and June 22nd. In the case of *A. hippocastanum*, also six NeemAzal-T/S applications could be realized with a time-interval of two weeks and three incidents of leaf collection on May 25th, June 22nd and July 19th. The initial application dates and the time-intervals between those incidents were suggested from the NeemAzal-T/S producing company Trifolio-M GmbH.

Then NeemAzal-T/S was diluted in water in a ratio 1:5 (20% NeemAzal-T/S). Two measuring cups (see Fig. 3.2a) assured exact proportions of compounds, before the mixture was properly stirred in the container of back-pack sprayer.

For the actual application process a 10 liter "Mesto" back-pack compression sprayer was used (see Fig. 3.2b). The spray mix was prepared individually for each tree, dependent on its diameter one meter above ground and applied to the tree stem (see Fig. 3.2c). For each cm in diameter, 5ml of NeemAzal-T/S was used, which is equivalent to 0,05g Azadirachtin per cm tree diameter.

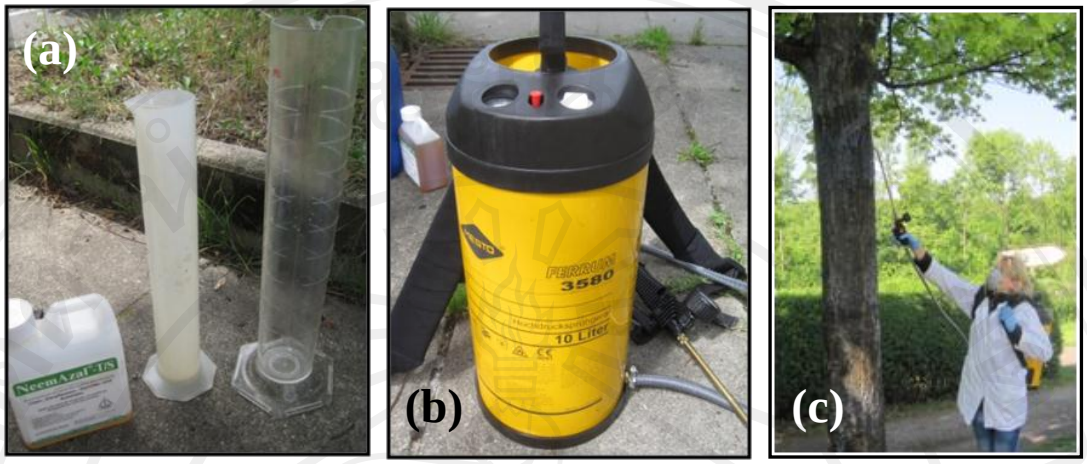

Figure 3.2: Measuring cups for exact preparation of spray mixture (a), used back-pack sprayer (b) and method of compound application (c)

The spray mixture was compressed to 2 bar, before applied individually to the tree trunks. An injection nozzle assured fine distribution of spray mixture. On none of the application dates rain or strong winds did disturb the accurate application. (see weather data in Appendix B)

The following tables show individual calculations of spray-mixture, dependent on the tree diameter. Differences to standard calculations are due to decimal places not shown at tree diameters.

Table 3.2 shows the selected Oak trees with their tree numbers, the treatment option (green color for treated trees and no color for control trees), the diameter in one meter above ground and the amounts of NeemAzal-T/S and water to formulate the spray mixture.

Table 3.2: Calculated amounts of NeemAzal-T/S spray mixture for treated Oak trees

NeemAzal-T/S research on Oak against OPM, 2011					
Tree #	Tree diameter in cm	NeemAzal in ml	H_2O in ml	spray mixture (20%) in ml	remarks
56	25	127	509	636	Neem
55	37	185	738	923	Neem
54	35	175	700	875	Neem
52	29	0	0	0	control
51	40	0	0	0	control
50	16	0	0	0	control
48	39	194	776	970	Neem
47	32	159	636	795	Neem
46	36	180	719	899	Neem
45	29	145	579	724	Neem
44	31	0	0	0	control
43	40	0	0	0	control
42	24	0	0	0	control
41	33	0	0	0	control

Regarding the Oak, seven trees for application with the NeemAzal-T/S mixture, and seven control samples were selected.

The following table (Tab. 3.3) shows these parameters for the Sycamore trees.

Table 3.3: Calculated amounts of spray mixture for treated Sycamore trees.

NeemAzal-T/S research on Platanus against SLB, 2011					
Tree #	Tree diameter in cm	NeemAzal in ml	H_2O in ml	spray mixture (20%) in ml	remarks
89	35	175	700	875	Neem
90	20	102	407	509	Neem
91	42	210	840	1050	Neem
92	40	200	802	1002	Neem
93	40	200	802	1002	Neem
97	26	0	0	0	control
98	48	0	0	0	control
99	23	0	0	0	control
101	22	0	0	0	control
104	43	0	0	0	control
108	41	204	814	1018	Neem
109	19	95	382	477	Neem
110	61	305	1222	1527	Neem
112	19	94	375	469	Neem

Regarding the Sycamore trees, nine trees for application with the NeemAzal-T/S mixture, and five control samples were selected.

The following table (Tab. 3.4) shows these parameters of treatment option, tree diameter and amount of NeemAzal-T/S and water for composition of spray mixture for the Horse chestnut trees.

Table 3.4: Calculated amounts of spray mixture for treated Horse chestnut trees.

NeemAzal-T/S research on Aesculus agianst HCL, 2011					
Tree #	Tree diameter in cm	NeemAzal in ml	H_2O in ml	spray mixture (20%) in ml	remarks
73	29	0	0	0	control
72	28	0	0	0	control
71	44	0	0	0	control
70	32	0	0	0	control
69	26	135	540	675	Neem
68	32	155	620	775	Neem
67	30	150	600	750	Neem
66	29	150	600	750	Neem
65	33	155	620	775	Neem
64	30	150	600	750	Neem
63	34	0	0	0	control
62	25	0	0	0	control
56				Pheromon trap	
46				Pheromon trap	

Regarding the Horse chestnut trees, six trees for the application of NeemAzal-T/S mixture, and six as control sample were selected. Two pheromone traps for HCL monitoring were placed in tree 46 and 56.

Accompanying the NeemAzal-T/S applications, frequent visits of visual observations were conducted and changes in pest population, behavior and symptoms reported. Pictures were also taken and findings documented.

Leaf samples had been collected with a one week delay to the latest application date, for the active ingredients being completely distributed within the plant. The leaf samples were taken for the Oak trees once, the Sycamore trees twice and the Horse chestnut trees three times.

To evaluate NeemAzal-T/S concentration levels distributed in the tree canopy, leaf samples from different layers (upper, central and lower) of the canopy had been taken. This had been realized during the leaf collection dates of June 22nd and July 19th, but not for May 25th.

On May 25th, leaves of all three tree species were collected only from a central and lower canopy layer. This was done with an extendable and manual tree pruning scissor reaching 3,5 meters in height. Figure 3.3 shows this tool.

Figure 3.3: Extended manual tree pruning scissor

Leaf collection on June 22nd and July 19th has been done in a climbing manner executed with arborist climbing gear. With this technique, leaf samples from the upper, center and lower canopy layer were collected (see Figure 3.4a).

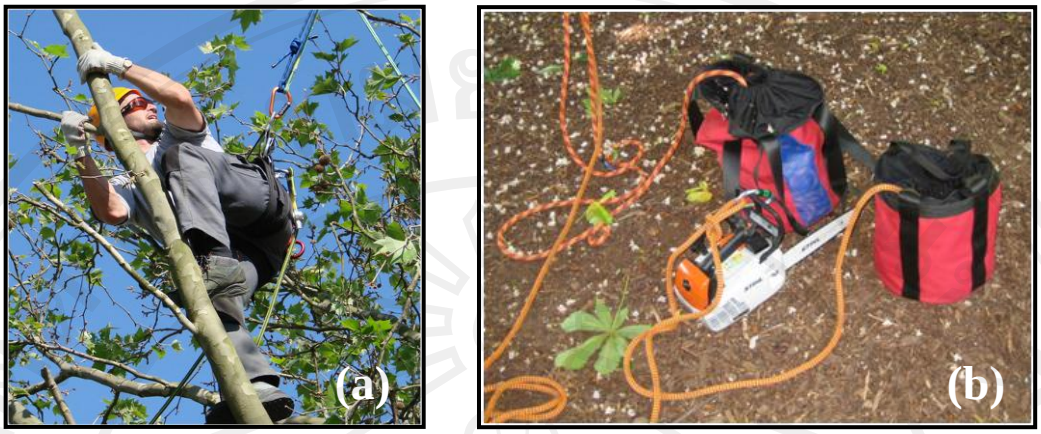

Figure 3.4: Climbing style for leaf collection (a) and parts of common climbing gear (b)

Leaf collection happened always on three treated trees as well as on three control trees, which were randomly selected. At the different canopy locations (top, center, low) always 10 leaves were collected and stored in plastic bags, which were marked beforehand. Information on plastic bags included the research topic, tree species, collection date, tree number, treatment option (treated or control) and canopy place.

On May 25th, leaves were collected on Oak, Sycamore and Horse chestnut on six trees (3 treated trees, 3 control trees) of each species from center and lower canopy layer making 3 times 12 units, which adds up to a total of 360 leaves collected.

On June 22nd, leaves were collected on Sycamore and Horse chestnut on six trees of each species from top, center and lower canopy layer making 2 times 18 units, which adds up to a total of 360 leaves collected.

On July 19th, leaves were collected only on Horse chestnut on six trees from top, center and lower canopy layer making 1 time 18 units, which adds up to a total of 180 leaves.

In conclusion 120 leaves were collected from Oak, 300 leaves from Sycamore and 480 from Horse chestnut, making 900 leaves altogether. Following table (Tab. 3.5) displays tree numbers of the randomly selected trees of each of the different tree species, leaf collection dates and treatment options.

Table 3.5: Selected tree numbers on different collection days and tree species

Date	Option	Oak	Sycamore	Horse chestnut
May 25	control	41, 43, 44	97, 98, 101	63, 70, 73
May 25	treated	46, 47, 48	90, 92, 108	65, 66, 69
June 22	control		98, 101, 104	70, 71, 72
June 22	treated		90, 91, 109	65, 67, 68
July 19	control			62, 70, 72
July 19	treated			64, 66, 69

The leaves of each collection day were refrigerated within the same day, with a temperature of minus 20°C and stored at the University of Hohenheim, institute for phytomedicine.

For observation of pest population density of the Horse chestnut leafminer (HCL) (*C. ohridella)* on *A. hippocastanum* triangular shaped pheromone traps were distributed in untreated trees located at a distance of more than 50m from the treated trees (Figure 3.5a and b).

Figure 3.5a, b: Sticky trap with stuck HCL moths (a) and delta trap placed in lower tree canopy (b)

The delta traps were placed in the lower canopy close to the main trunks of tree 46 and tree 56 above normal reaching height at about 3m above ground. The HCL were counted in the traps on a regular basis in addition to the frequent visual observations and collection of leaf samples. The sticky foil has been changed several times as well as the pheromone lures to retain the attractiveness of the traps. HCL captures with peak emergence were recorded (see Appendix A 2 for detailed data).

3.3 Procedural method for analysis

The different circumstances for each tree species initiated specific core areas of analytical emphasis, which caused a variation in the kind and method of the data analysis being carried out.

3.3.1 Visual tree observation

The rating of the different tree species happened on a regular basis in the field. (see Appendix D for dates and collected data).

During the visits following parameters were observed:

General tree condition, pest occurrence, degree of infestation through target pest calculation via nest counts or pheromone trap supported monitoring, furthermore observation of other organisms found on the tree.

Only in the case of the Horse chestnut trees, pest infestation was monitored with pheromone traps. The result of this visual observations were notated and later displayed in excel charts. The results of the visual estimations were also statistically tested and assessed through JMP analysis.

3.3.2 Method of tree-leaf analysis

To analyze the tree leaves collected and refrigerated, always three leaves of each unit were randomly selected and placed into a named plastic container. Those three leaves were then scrutinized in detail in laboratory research work. To assure objective and clear recording of analytical data, an analysis template containing all the necessary data had been created.

Following chart (Tab. 3.6) shows the fill in form used for Horse chestnut, to gather all necessary data for later analysis. Next to the general information like tree species, treatment option and collection date, the specific data of each leaf mine, their number and size as well as the corresponding larva with their head capsule diameter were recorded.

Table 3.6: Fill in form for analysis on Horse chestnut leaves

Tree species _____ Treatment option _____ Leaf collection date _____

Tree #	Canopy place	Leaf #	Finger #	Mine #	Size of Mine	Larvae #	Head Capsule	Remarks

To avoid confusion or mistakes, the analysis always followed a specific course of action. For example in the case of Horse chestnut leaf analysis, where the leaves show five to seven fingers, the recording of leaf-mines happened always from the very first left finger of leaf petiole starting at the inside towards leaf margin to the last finger on the right of leaf petiole.

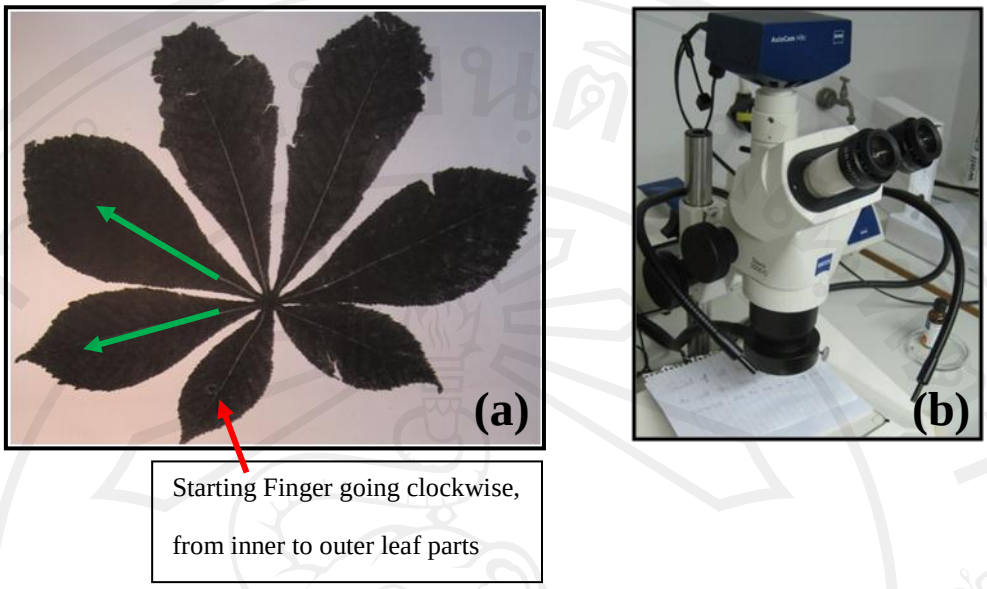

Figure 3.6: Course of action during HCL analysis (a) and used binocular (b)

Leaf mines were detected and larvae laid open through observation of the leaf surface with a binocular (Stemi 2000-C, from Zeiss, see Fig. 3.6b). Transmitted to a life screen via an Axio Cam from Zeiss, the head capsule of the open laid larvae was measured, and together with the mine size noted in the analysis template form (see Tab. 3.6). Examples for information recorded in the analysis template were the following: old mine with already hatched pupa, or pathogens on larvae, larvae in pupa stage, etc. These findings and remarks are accompanied by life screen pictures taken and documented. Results of this analysis are compiled in the database (see Appendix A 3), from where findings on Horse chestnut and statistical analysis derive.

Furthermore an analysis of total leaf area compared to total mine area on Horse chestnut had been conducted. For this analysis, always three leaves of each of the leaf collection days, of each canopy layer and treatment option (treated or control) were randomly selected and glued onto a sheet of paper.

One square meter of this paper weighs 80 grams. The leaves glued to the paper were then duplicated in a reprint machine and cut out with a scissor to its original leaf form.

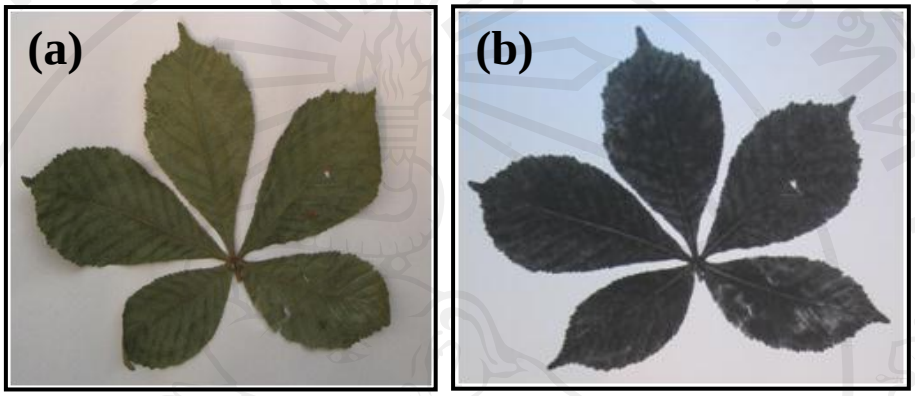

Figure 3.7: Original leaf glued to a paper-sheet (a) and blueprint of same Horse chestnut leaf (b), before cut-out with a scissor

To consider the weight of the print ink, three plain A4 paper sheets were weighted, and then printed to be covered with ink on one side completely and weighted again. The average difference of weight, (expressed through the ink correction factor 1,045) was then used for further considerations. The cut out leaf shape weights were determined with a digital advanced precision scale from OHAUS and multiplied with the ink correction factor. At this stage, the ink on the leaf forms was considered, as if they were covered with ink to 100%, but only 80% of the leaf shape weights with ink correction were finally considered and served as the basis to investigate the actual leaf area of each leaf unit (paper sheets with 80 g/m²). The leaf areas compared with the mine areas, which were determined in earlier analysis, gave the percentage with which the leaves are mine-covered from HCL.

Visual analyses on Oak and Sycamore tree leaves happened as well, but were not done in the same detail as done with the Horse chestnut leaves. This, of course, was founded in the fact that the OPM, the insect pest affiliated with the Oak leaves, does not live parts of their life-cycle within the tree leaf, and in the case of Sycamore, no SLB had been observed during these research investigations.

3.3.3 Method of Azadirachtin analysis

To evaluate effectiveness of the general concept of spot stem application of NeemAzal-T/S and approve systemic distribution within the tree, tree leaf analysis for Azadirachtin detection were conducted. To calibrate measurements, an Azadirachtin standard had been used.

HPLC translates high performance liquid chromatography and is an analytical, preparative and chromatographically method for dividing, identifying and quantifying chemical substances. To prepare leaf samples for high pressure liquid chromatography, mass spectrometry (HPLC-MS) analysis, the following procedures had been executed: Leaves have been chopped into small pieces, and 2gr. of leaf material were homogenized in a 100 ml centrifugal capillary tube with 25ml of Acetonitril. The substance was filtered with a wrinkle-filter into a 100ml round bottom flask, before rinsed with Acetonitril and dried in a vacuum-rotation-evaporator with 180bars. Assimilation of residue into an Eppendorf-reaction-vial with 1ml and 0,5ml of Acetonitril, was a further step, before Acetonitril was evaporated again within a thermal block of 60°C and anew assimilation of residue in 1ml of 3/7 Acetonitril/water mixture. The substance was centrifuged for 5 min with 12.000 rpm

and once again placed into an Eppendorf-reaction vial. The assay was stored in the refrigerator over-night and finally centrifuged (again for 5 min with 12.000 rpm), before applied to the HPLC-MS analysis.

3.4 Statistical analysis

All data obtained were subjected to statistical analysis using JMP® 7.0.2 software (SAS Institute Inc., Cary, NC, USA). The respective statistical procedures are provided in the legends of the figures and tables. Outliers were excluded using Jackknife- or Mahalanobis-method.